Soil Drenching of *Soluanum tuberosum* Plants with *Trichoderma atroviride* or *Aspergillus tubingensis* Spores induces Natural Defence Pathways: A Metabolomic Study.

Introduction

Solanum tuberosum, commonly known as the potato, is staple crop across the world. Despite the global re-emergence of *Phytophthora infestans* (*P.inf.*) (Fry, W.E. *et al.* 2015), the cause of late blight in potatoes, no effective biocontrol agents or reliably resistance potato varieties have been developed (Coomber, A. *et al.* 2024). As environmental and health concerns about the use of broad-spectrum chemicals for crop treatment grow, research into sustainable biocontrol agents is advancing rapidly. Biocontrol agents have a wide ranging of modes of action in terms plant protection such as induction of plant defences or direct antagonism of the pathogen (Sheoran, A.R. *et al.* 2025 & de Vrieze, M *et al.* 2019). Although the use of bacterial biocontrol agents has gained a lot of attention in recent years (Ebrahimi-Zarandi M *et al.* 2022), the use of the fungal strain to protect our crops also shows promise (Abada, K. A. *et al.* 2025). Certain species of the Trichoderma and Aspergillus genera's have been shown to directly antagonise *P.inf.* and induce disease resistance in their plant hosts (Madlhophe, S. *et al.* 2025 & Wang, S. *et al.* 2024). Furthermore, research have shown that fungi from the *Aspergillus* or *Trichoderma* genera are able to both the phyllosphere and rhizosphere of various plants species highlight their broad establishment ranges (Bao, L. *et al.* 2020). More specifically in *Solanacea* plants, applying a *Trichoderma* strain to tomato seeds led to colonisation of root, shoot and leave tissues (Agbessenou, K. *et al.* 2022).

While biocontrol agents are more environmentally friendly than copper, still used in organic farming, introducing non-native microbes poses its own risks, including negative impacts upon establishment and dispersal and unpredictable direct and indirect non-target effects (Loomans, A.J.M. 2021). The use of native microbes as biocontrol agents however does mitigate those risks. Furthermore, as our understanding of host-microbiota interactions evolves so does research into the use of native biocontrol agents to protect crop plants. This has led to 2 hypotheses upon which this research was based. Firstly, our crop plants harbour the organisms necessary to directly antagonize their pathogens. In various examples, *Pseudomonas* strains isolated from potato plants have shown great promise at directly antagonising various developmental stage of *P.inf.* (Hunziker, L. *et al.* 2015 & de Vrieze, M. *et al.* 2018). The same trends were also true for *Trichoderma* strains isolated from potato plants (Yao, Y. *et al.* 2016) and *Aspergillus* strains from other plant species (Wang, S. *et al.* 2024). In grapevine varieties naturally resistant to *Botrytis cinerea,* higher abundances of microbes antagonistic towards said pathogen have been observed suggesting contributions of the native microbiota to

resistant phenotypes by direct antagonism of the pathogen in question (Richard, T. *et al*. 2016).

Secondly, our crops plants harbour organism capable of inducing resistance to the pathogens that plague them. For example, the application of strains of *Trichoderma Atroviride* isolated from potato plants have been shown to induce systemic resistance against late blight in potato plants (Purwantisari, S., *et al*. 2018). *Aspergillus* strains isolated from soybean plants have also been shown to protect the plant host against the devastating nematode pathogen *Heterodera glycines* by inducing resistance via the stimulation of hydrogen peroxide activity in the roots (Jin, N. *et al*. 2019).

Furthermore, evidence in *Arabidopsis thaliana* also shows that plants undergoing an attack by a pathogen can shift their rhizosphere microbiome composition to recruit beneficial microbes which synergistically protected the plants against the pathogen (Berendsen, R.L. *et al*. 2018). This suggests that plants can be "vaccinated" against certain pathogens.

The isolation and use of the *Trichoderma* and *Aspergillus* strain in this study are based on the above hypotheses and the observation made in the model species *Arabidopsis thaliana*.

The *Trichoderma* strain used in this study was isolated from the rhizosphere of potatoes having undergone "vaccination" with *P.inf*. and was shown to increase in abundance following *P.inf*. infection on leaves. The *Aspergillus* strain was isolated from the leaves of potato plant showing resistance to *P.inf*. infection and which were potted in soils which previously contained 2 generations of plants having undergone "vaccination". Furthemore, both strains have been shown to inhibit the mycelial growth of *P.inf*. in dual-assay conditions (Pichon, V. *et al*. unpublished data).

In this study, we investigated the metabolic responses of *Solanum tuberosum* plants having undergone weekly soil drenching since emergence with *Trichoderma atroviride* and *Aspergillus tubingensis* spores.

For our first aim, we wanted to understand how the plants react to the fungal drenching treatments. Secondly, we wanted to know whether drenching the soil of recently emerged potato plants permits the establishment of the fungi themselves or deposition of their metabolites in the phyllosphere of *Solanum tuberosum* plants. To this end, untargeted metabolomic profiling was performed on both treated and untreated potato leaves, as well as on the fungal cultures themselves. This allowed us to compare the metabolic profiles of each condition and to distinguish plant-derived putative metabolites from those originating from the fungi. The objective of this dual approach was to identify metabolic signatures that could indicate the induction of a systemic defence response in the plant and/or the uptake and presence of fungal metabolites in or on plant tissues which could protect the plant by direct antagonism of the pathogen in question. In addition, we spent time analysing clusters of features specific to either fungus, in hopes of gaining a better understanding of their metabolic profiles.

Materials & Methods

Fungal strain isolation

To isolate beneficial microbial strains, potted *Solanum tuberosum* (cv. Bintje) plants were artificially infected with *Phytophthora infestans*. The same soil was reused over three consecutive plant generations, with each generation undergoing artificial infection. At each stage (pre- and post-infection), the rhizospheric and phyllospheric microbiomes were analyzed using next-generation sequencing (NGS) to assess alpha diversity. Microbes were isolated from the rhizosphere and phyllosphere of plants that showed increased resistance following repeated exposure. All phyllospheric microbes from resistant plants, including the *Trichoderma atroviride* strain in question were retained. Rhizospheric microbes that increased in abundance after infection, such as the *Aspergillus tubingensis* strain in question were also retained. (N.B. trich_1 refers to *Trichoderma atroviride,* trich_2 refers to *Apsergillus tubingensis* which was thought to be a *Trichoderma spp*. at the time of sampling)

Growth conditions

The plants were potted and grown in greenhouse conditions. Since emergence the surrounding soil (3cm around the shoot) was drenched 1/week with 10mL of *Trichoderma atroviride* or *Aspergillus tubingensis* spores at 10^6 sp/mL. The control plants were devoid of this treatment. All plants were watered 2/week and were 10-weeks old at the time of sampling. The fungi were 3-days old at sampling time and were grown on PDA-agar 15g/L plates.

Sample collection and preparation

12 healthy leaflets from the 4rd to 6th generation leaves Erika potatoes were collected in the greenhouse using a sterilised scalpel to cut entire leaflets. These were placed in coffee filters and then in pierced 50mL falcon tube before submerging the samples in liquid nitrogen. These were then lipolyzed and kept for 48h before samples preparation.
~50mg of leaflet fragments were placed in 2mL Eppendorf tubes. Sterile stainless-steel beads and 1.5 mL of extraction solvent consisting of 80% methanol, 20% water, and 0.1% formic acid were added to the tubes. Samples were then homogenized using a Retsch tissue lyser for 3 minutes. After homogenization, the samples were centrifuged, and approximately 1 mL of the supernatant was transferred into glass vials suitable for mass spectrometry analysis.

LC-MS analysis

Samples were analyzed using liquid chromatography–tandem mass spectrometry (LC-MS/MS). Initial separation was performed using an ultra-high-performance liquid chromatography (UHPLC) instrument (ThermoFisher megaUPLC 3000 xyz, Thermo Fisher Scientific), which offers greater speed and sensitivity compared to conventional high-performance liquid chromatography (HPLC) systems. Molecules were separated on a reverse-phase column based on polarity. The eluate was then introduced into a mass spectrometer (ThermoFisher Orbi Qtrap Quadrupole Hyper 5000 xyz), where MS/MS analysis was used to quantify metabolite profiles.
The details for the LC and MS conditions can be found in the links bellow:

1. LC Conditions
2. MS Conditions

Data treatment

The raw LC-MS/MS data files underwent initial conversion into a compatible format via ProteoWizard for subsequent analysis in MZmine (v4.6.1). Data processing involved a sequential workflow to extract and organize molecular features. Peak detection identified true spectral signals while filtering background noise, followed by chromatogram construction to generate extracted ion chromatograms (XIC/EIC) for tracking ion intensity across retention time. Peak deconvolution resolved overlapping chromatographic peaks to isolate individual compounds, while isotope grouping consolidated isotopic variants into monoisotopic mass features. Finally, feature alignment harmonized detected features across all samples to enable comparative abundance analysis.
Processed data and metadata were imported into Cytoscape (v3.10.3) to construct a molecular network, visualizing the chemical space of detected metabolites. For annotation, GNPS facilitated spectral library matching and network analysis, while SIRIUS, Canopus, and ISDB predicted molecular formulas and compound classes. Structural visualization within Cytoscape was optimized using the Cheminformatics Tools plugin.
N.B.: Full parameter details for data processing are available here.

Results

In total we obtained 7132 features, available here: final feature list.

Does Fungal Soil Drenching Lead to Phyllosphere Establishment?

Despite subjecting plants to fungal drenching for up to eight weeks, analysis of the putative metabolomes (Fig. 1) showed no significant differences in diversity or abundance between control and treated samples. This indicates that neither the fungi (*Trichoderma atroviride* or *Aspergillus tubingensis*) nor their metabolites successfully established a detectable presence on the leaves following soil application. Further evidence supporting this conclusion was obtained by analysing the spectrally clustered molecular network of leaflet samples in Cytoscape (Fig. 2). No nodes representing putative metabolites uniquely shared between treated leaves and the fungal sample, excluding those also present in the control, were found. This result further bolsters our confidence in rejecting the initial hypothesis, confirming that soil application of fungal spores does not lead to detectable fungal establishment or metabolite production on potato leaves following plant emergence.

Figure 1: **Metabolite annotation overview**. The size of boxes is proportional the individual count of metabolites pertaining to said class

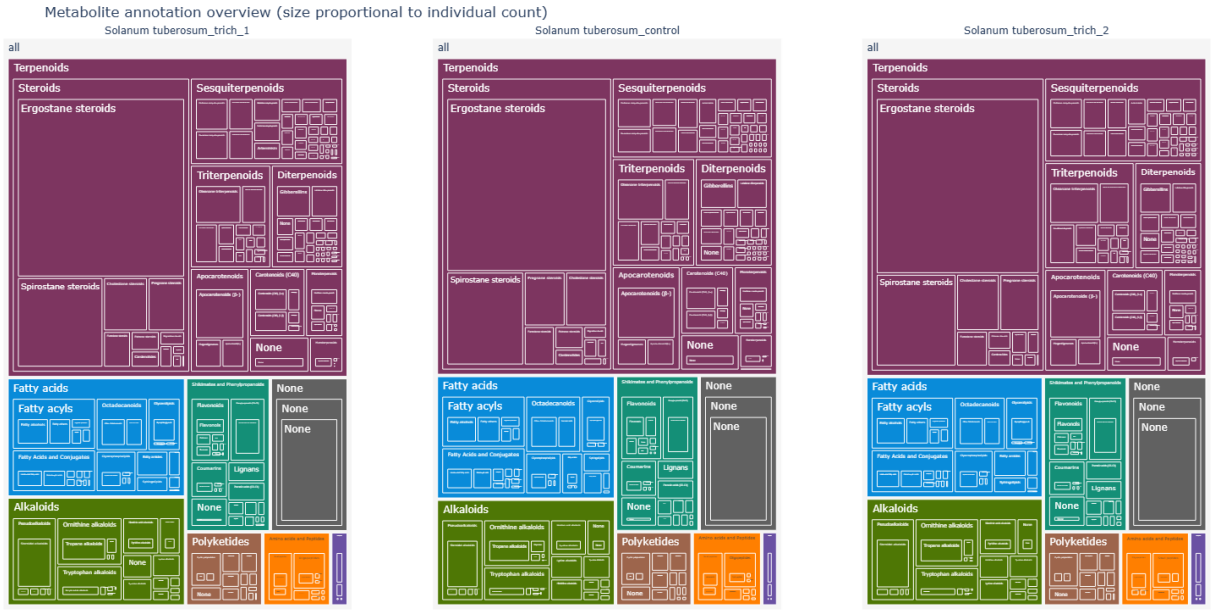
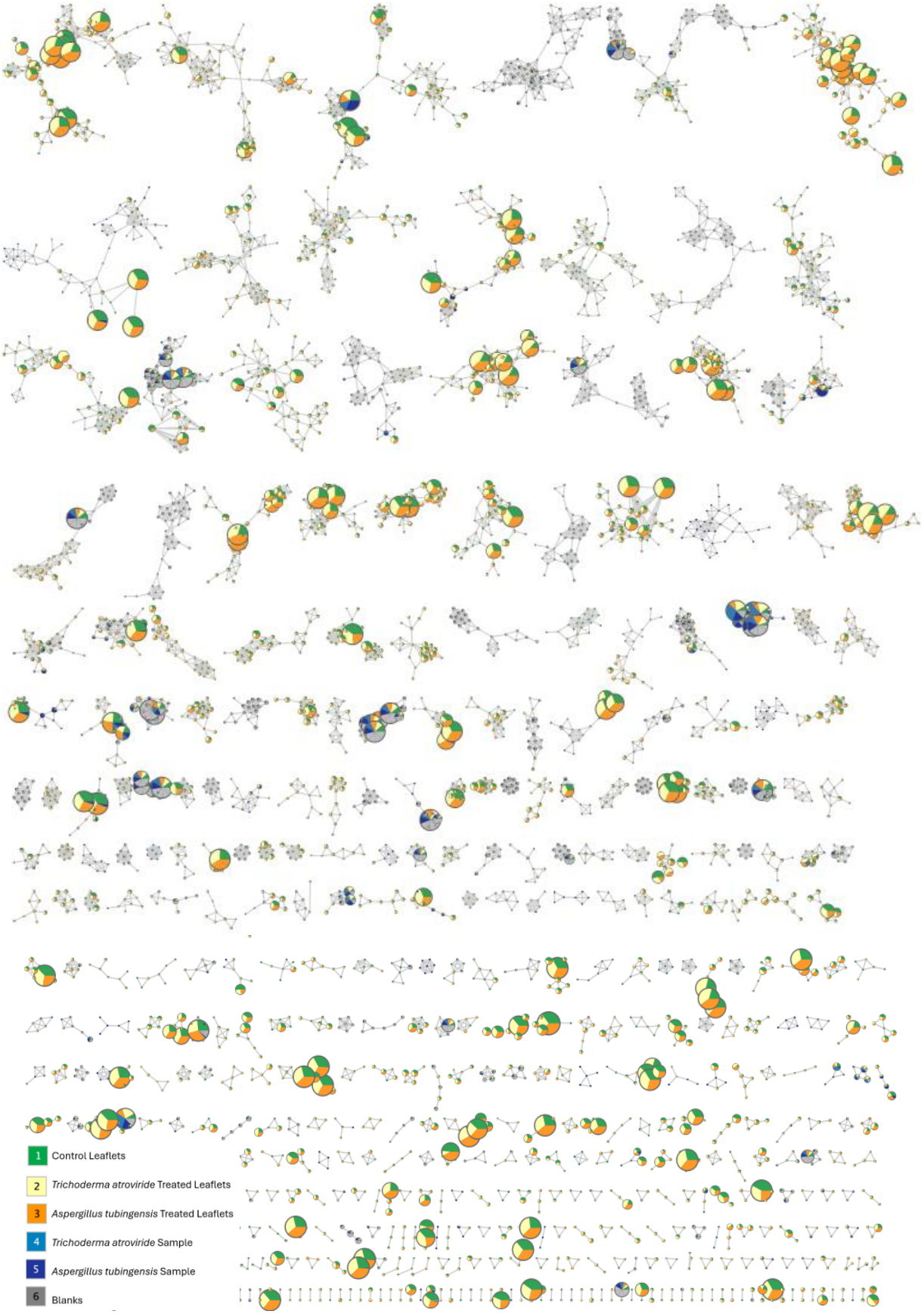


Figure 2: **Molecular Network of Treated and Non-Treated Leaves.** The size of the pie charts corresponds to the relative abundance of these features in treated leaves. All cluster of up to 3 features are shown.



How were leaflets of treated plants affected by soil drenching with fungal spores?

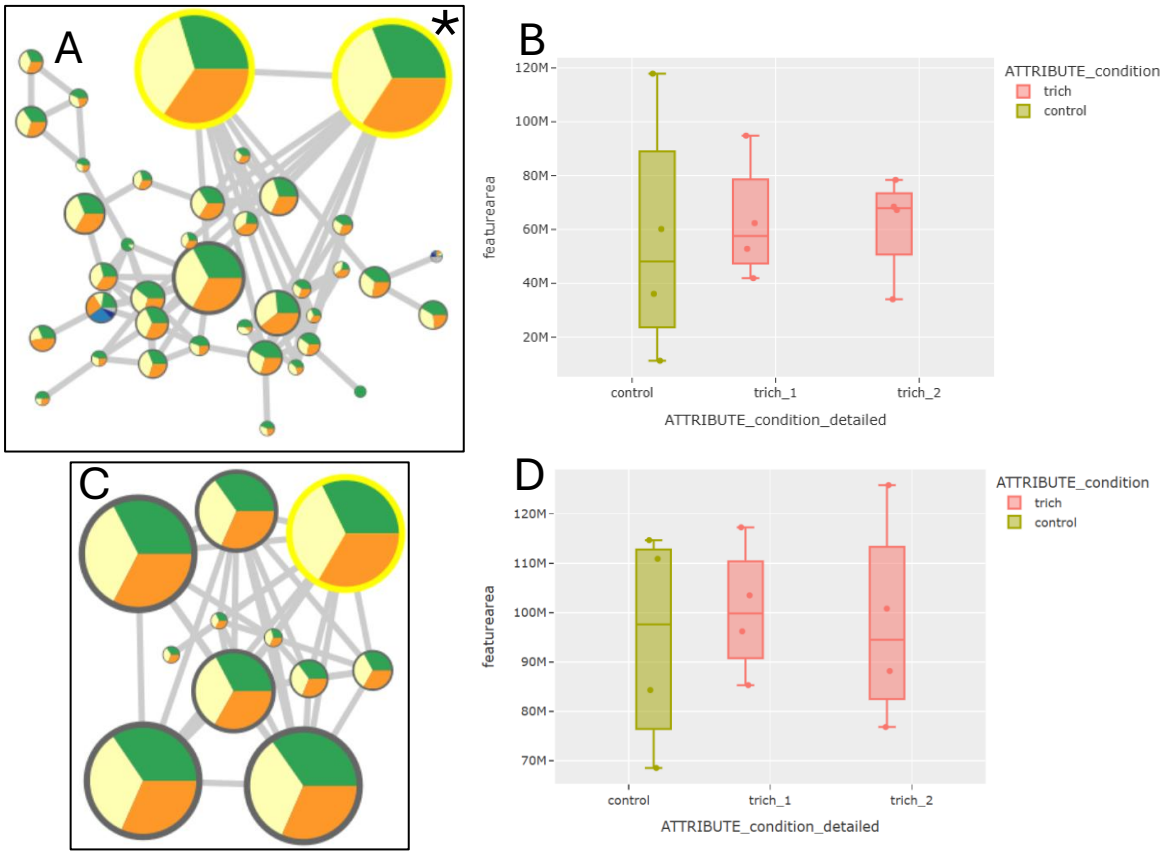


Figure 3: **Metabolite Features Equally Abundant in Treated and Control Leaves.** (A) Features highlighted in yellow are putatively identified as Apramide A and Apramide C. (B) The relative abundance of Apramide C (*) in the two treated groups and one control group is shown in the box plot. (C) Additional features within the cluster are identified as neutral glycosphingolipids. (D) The relative abundance of the glycosphingolipids highlighted in yellow in panel (C) is displayed here. Pie chart size reflects the relative abundance of each feature in control leaf samples.

Many features are found in equal abundance in control and treated plants. Notably natural lipids constituting plant cell walls in plants such as neutral glycosphingolipids (Fig 3, C) who's relative abundance would not expectedly change upon soil drenching with fungal spores.

More interestingly certain features of prokaryotic provenance (*Cyanobacteria spp.* or *Proteobacteria spp.* according to CANOPUS) such as the anti-microbial compounds Apramide A, Apramide C, Ogipeptin B, or are also equally abundant suggesting that the fungal drenching treatment did not significantly alter the phyllospheric microbiota composition of such phyla (Fig 3, A).

Despite this there were many putative metabolites which significantly variety in abundance between the control and treated plants (Fig 4).

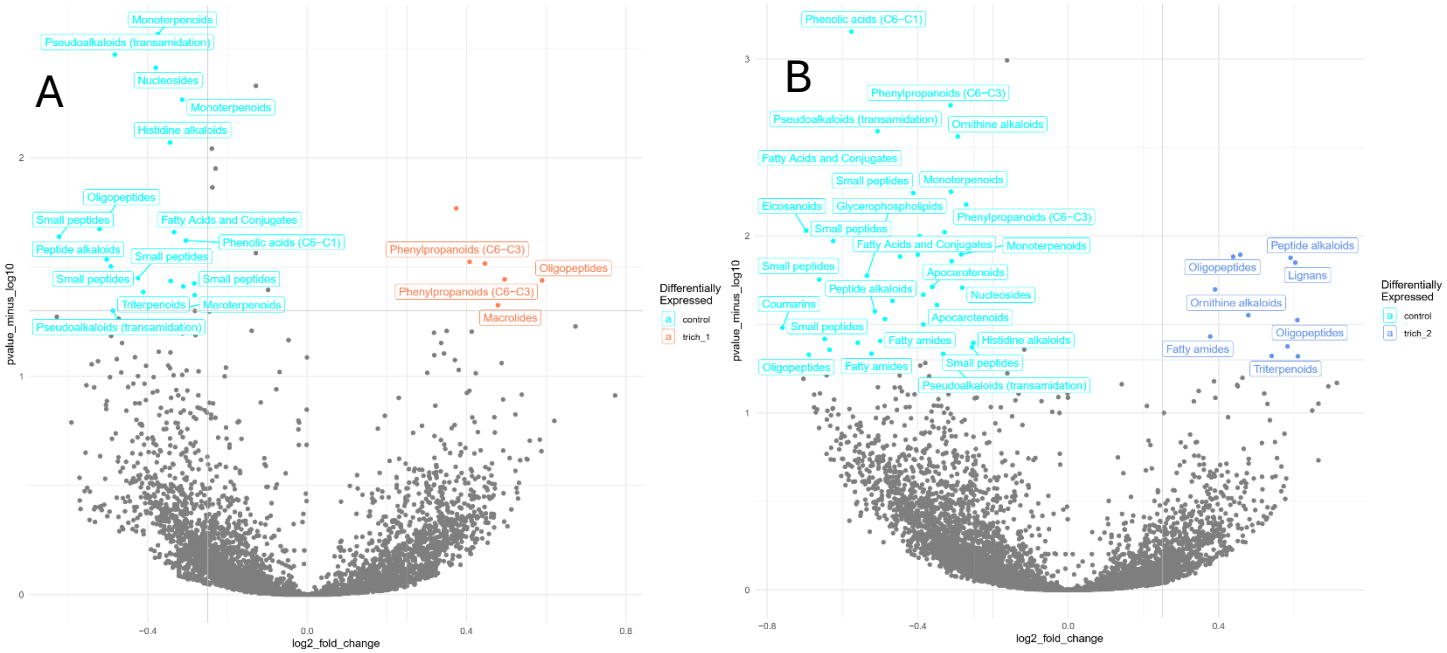


Figure 4: **Differentially Abundant Metabolite Superclasses in Treated Versus Control Leaflets.** Metabolite superclass annotation was performed using the CANOPUS database. (A) Superclasses that are significantly more abundant in *Trichoderma atroviride*-treated leaflets are highlighted in orange, while those significantly less abundant in control leaflets are shown in cyan. (B) Superclasses that are significantly more abundant in *Aspergillus tubingensis*-treated leaflets are indicated in orange, and those significantly less abundant in control leaflets are displayed in cyan.

Higher abundance of Saponin features in treated plants

In plants, saponins (Fig. 5) play a key role in natural defence against microbes, insects, and fungi. They are composed of two parts: a hydrophilic sugar chain and a hydrophobic aglycone nucleus, often based on a steroid or triterpene structure. This unique amphipathic composition allows them to interact with and disrupt the membranes of pathogens, contributing to their antimicrobial action. Beyond their role in plants, saponins also exhibit a wide range of biological activities in animals and humans, including antifungal, antibacterial, antiviral, and anti-inflammatory effects (Trdà, L. *et al*. 2019).

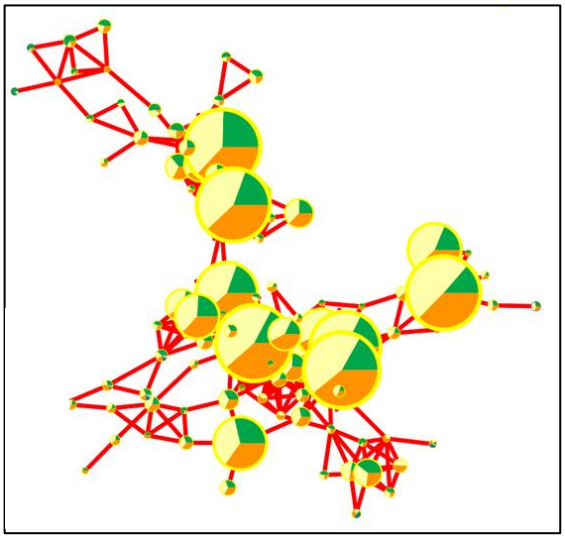
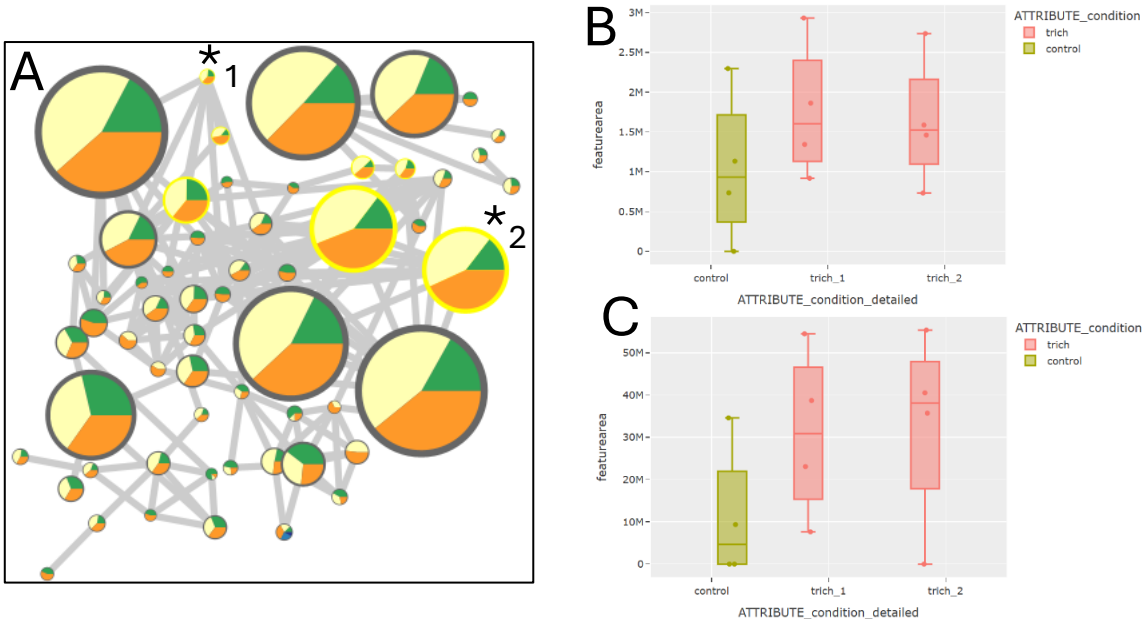


Figure 5: **Steroidal glycoalkaloids and saponin-like compounds are enriched in treated leaflets.**
Pie chart size reflects the relative abundance of each feature in treated leaf samples.

The cluster presented in Figure 6A includes multiple features corresponding to saponin precursors or derivatives, such as the annotated compounds Tuberoside D and Tuberoside C (Fig. 6B). This cluster also contains other putatively identified molecules implicated in natural defense pathways, including α-chaconine (Fig. 6C). Notably, these features are more abundant in treated leaflets particularly those exposed to *Trichoderma* compared to untreated control plants (Fig. 6A/B). Tuberosides are steroidal saponins commonly isolated from *Solanum tuberosum* and play a direct role in plant defense by disrupting pathogen membranes (Li, Y. et al., 2023). However, while these compounds are effective against pathogens, they can also be toxic to humans and animals, highlighting the importance of carefully balancing their levels in efforts to develop improved potato varieties (Liu, Y. et al., 2024).



D

shared name	isdb_structure_nameTraditional	isdb_npc_pathway	isdb_npc_superclass	isdb_npc_class
4022	methyl (E)-5-[(1S,4aR,6R,7S,8aR)-6,7-di...	Terpenoids	Diterpenoids	Norlabdane diterpenoids
4339	alpha-Chaconine	Alkaloids	Pseudoalkaloids	Steroidal alkaloids
4411	alpha-Chaconine	Alkaloids	Pseudoalkaloids	Steroidal alkaloids
4585	alpha-Chaconine	Alkaloids	Pseudoalkaloids	Steroidal alkaloids
5120	Tuberoside D	Terpenoids	Steroids	Spirostane steroids
4697	Tuberoside C	Terpenoids	Steroids	Spirostane steroids
5286	Tuberoside C	Terpenoids	Steroids	Spirostane steroids
5363	Tuberoside C	Terpenoids	Steroids	Spirostane steroids
4807	N-[(2R,6S)-6-[(3S,5S,8R,9S,10S,13S,14S...	Terpenoids	Steroids	Cholestane steroids
2346	Gibberellin A44	Terpenoids	Diterpenoids	Gibberellins
5409	Cilistol pl	Terpenoids	Steroids	Ergostane steroids
4768	Cilistol pl	Terpenoids	Steroids	Ergostane steroids
5138	Cilistol pl	Terpenoids	Steroids	Ergostane steroids
3302	9-Oxononanoic acid	Fatty acids	Fatty Acids and Conjugates	Oxo fatty acids
3314	9-Oxononanoic acid	Fatty acids	Fatty Acids and Conjugates	Oxo fatty acids
3545	9-Oxononanoic acid	Polyketides	Cyclic polyketides	4-pyrone derivatives
4262	8-Methylpodopyrone	Polyketides	Cyclic polyketides	4-pyrone derivatives
4329	8-Methylpodopyrone	Polyketides	Cyclic polyketides	4-pyrone derivatives

Figure 6 : **Natural defence related compounds are enriched in treated plants.** (A) The highlighted nodes of the cluster correspond to the putative α-chaconine or tuberoside features. Pie chart size reflects the relative abundance of each feature in treated leaf samples. (B) The relative abundance of a putative α-chaconine feature ($*_1$) in the two treated groups and one control group is shown in the box plot. (C) The relative abundance of an putative Tuberoside-C feature ($*_1$) in the two treated groups and one control group is shown in the box plot. (D) A list of putative features, annotated in ISDB, more abundant in treated plants is shown.

The annotated compounds in this cluster (Fig. 6D) include molecules structurally related to several classes, such as spirostanes, cholestanes, ergostane steroids, and steroidal alkaloids. These structural classes represent the typical aglycone cores from which many saponins are derived (Duan, L. et al., 2023). Spirostane steroids, characterized by an additional fifth ring that results in a hexacyclic structure, are commonly found among plant steroidal saponins, especially within the Solanaceae family (Xu, Y. et al., 2020). Cholestane steroids, derived from cholesterol, serve as precursors in the biosynthesis of steroidal saponins (Milner, S. E. et al., 2011). Ergostane steroids, closely related to cholestanes but with additional modifications, are frequently found in fungi and certain plants (Glotter, E., 1991). Steroidal alkaloids, such as α-solanine and α-chaconine, are nitrogen-containing compounds prevalent in the Solanaceae family, including potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicum*), and play a significant role in the chemical defense mechanisms of these plants (Wang, Y. et al., 2023). Furthermore, studies have shown that induction of plant defences, more specifically the biosynthesis of saponin-related compounds is a common theme in response to fungal exposure (Moses, T. *et al*. 2021). As saponins are known to disrupt fungal membranes, their accumulation in treated plants may represent an adaptive biochemical strategy to counteract infection (Shakeel, A. *et al*. 2025). However, this response is unlikely to be directed specifically against *Aspergillus tubingensis* or *Trichoderma atroviride*, as these fungal species have already been identified as natural members of the potato microbiome and the latter has been shown to induce systemic resistance against late blight in potato with no phytotoxic effects reported (Purwantisari, S., *et al*. 2018).

Recent studies have demonstrated that some glycoalkaloids such as α-chaconine and α-solanine have anti-oomycte properties, notably against *Phytophthora infestans,* the pathogen responsible for potato late blight. For example, research has shown that compounds such as α-solanine can inhibit the motility of *P. infestans* zoospores, thus reducing the probability of infection. This inhibitory effect is attributed to their ability to damage fungal-like cell membranes, resulting in increased permeability and cell death (Chacón , M. G. *et al*. 2023).

Monolinolein: Proven effective against *Phytophthora infestans*

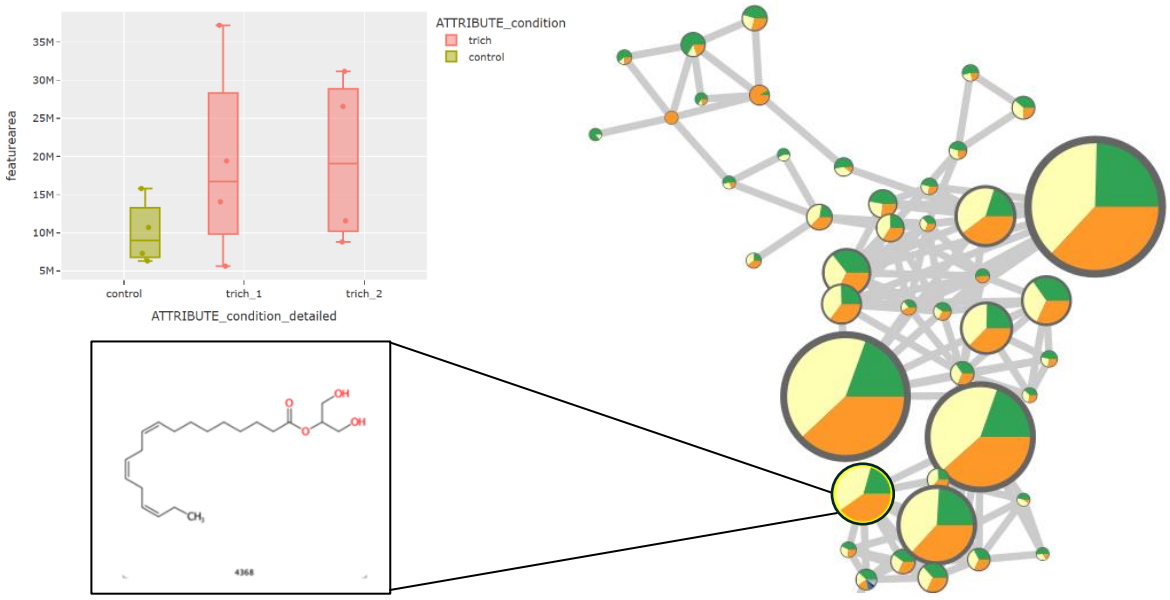


Figure 7: **Monolinolein: a phenolic compound enriched in treated leaflets.** Molecular network highlighting monolinolein (black circle). Pie chart size reflects the relative abundance of each feature in treated leaf samples. The structure of monolinolein (monoacylglycerol of linoleic acid) along with a boxplot showing a monolinolein abundance in *Trichoderma* and *Aspergillus* treated plants compared to control plants.

Another notable compound annotated within this saponin-associated cluster was monolinolein, which was more abundant in plants treated with *Aspergillus* and *Trichoderma*. Monolinolein is a naturally occurring lipid molecule formed by the esterification of one linoleic acid molecule (an unsaturated fatty acid) to a glycerol backbone. As reported by Stoessl et al. (1980), monolinolein significantly inhibits the germination of *Phytophthora infestans* zoospores and is commercially available (Monolinolein, Larodan, CAS number: 26545-74-4). Its high efficacy at low concentrations suggesting that monolinolein plays a key role in plant chemical defence by blocking a critical step in the pathogen's infection cycle.

Phloretin: A compound released in response to stress

Phloretin (Fig. 8) is a dihydrochalcone compound widely present in various fruits and vegetables, with particularly high concentrations in apple and apricot trees. Known for its anti-inflammatory, antioxidant, and anticancer properties in humans (Zhang, Y. et al., 2022), phloretin also demonstrates significant biological activity in plant defence. Notably, as reported in the article "Control Efficacy of Phloretin Isolated from Apple Fruits Against Several Plant Diseases" (Shim, S.-H. et al., 2010), phloretin, a phenolic compound extracted from apple fruits, exhibits strong antifungal effects. In *in vivo* trials, a preventive application of phloretin (500 µg/ml) to tomato seedlings significantly inhibited late blight development. Plants treated one day before inoculation showed a marked reduction in downy mildew symptoms compared to untreated controls. In the context of our study, the application of *Trichoderma atroviride* and *Aspergillus tubingensis* appears to increase the abundance of phloretin in potato plants, suggesting a potential role for this compound in induced systemic resistance and plant defence responses.

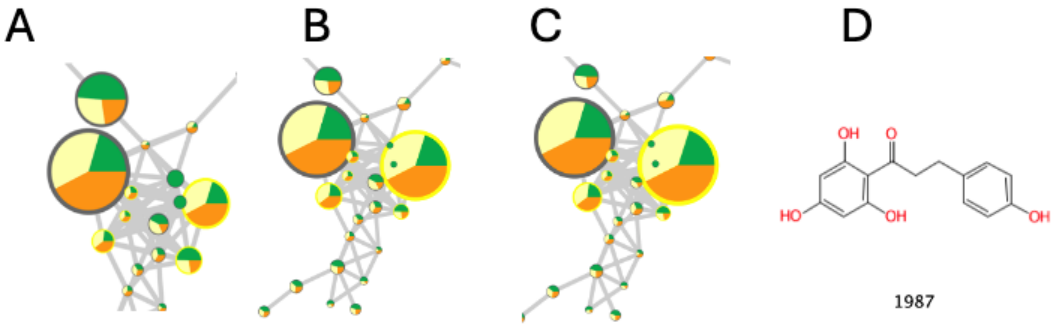
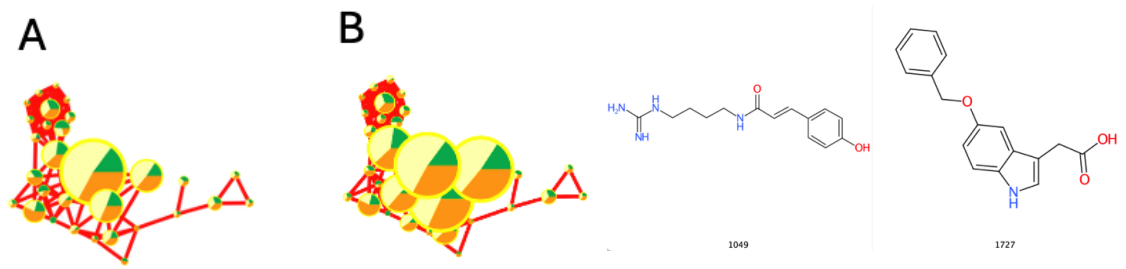


Figure 8: **Phloretin abundance varies amongst treatments.** Although phloretin is a phenolic compound (not a saponin), its spectral profile grouped it within the cluster containing several saponin-like features. (A) Abundance distribution of the cluster in control samples (B) Abundance distribution of the cluster of *Trichoderma atroviride*-treated leaves; (C) Abundance distribution of the cluster of *Aspergillus tubingensis*-treated leaves; (D) Chemical structure of phloretin (feature 1987), a phenolic compound previously reported to inhibit *Phytophthora infestans*.

Plant Defence-Associated Phenolic Acids

One cluster shows an overall increase in abundance in plants treated with *Trichoderma* compared to untreated controls (Fig. 9A/B). This cluster contains several features annotated as "coumaric acids" and derivatives" with CANOPUS subclass probabilities exceeding 0.95. These compounds belong to the family of phenolic acids, a group of specialized plant metabolites known for their role in stress and defence responses. (Zaman, A. *et al.* 2023). From a structural point of view (Fig. 9), coumaric acid and its derivatives are considered phenolic compounds, characterized by a hydroxyl group attached to an aromatic ring (Kemat, N. *et al.* 2020).



canopus_ClassyFire#superclass	canopus_ClassyFire#subclass Probability	canopus_ClassyFire#level 5
Phenylpropanoids and polyketides	0.949	Coumaric acids and derivatives
Phenylpropanoids and polyketides	0.925	Coumaric acids and derivatives
Phenylpropanoids and polyketides	0.957	Coumaric acids and derivatives
Phenylpropanoids and polyketides	0.96	Coumaric acids and derivatives
Phenylpropanoids and polyketides	0.98	Coumaric acids and derivatives
Phenylpropanoids and polyketides	0.989	Coumaric acids and derivatives

Figure 9: **Cluster of phenolic compounds.** These compounds are primarily annotated as *coumaric acid and cinnamic acids derivates*, known to be involved in plant defence responses. (A) Relative abundance in control plants; (B) Relative abundance in *Trichoderma*-treated plants. The structure of 2 phenols compounds annotated as coumaric acid compounds are shown on the right.

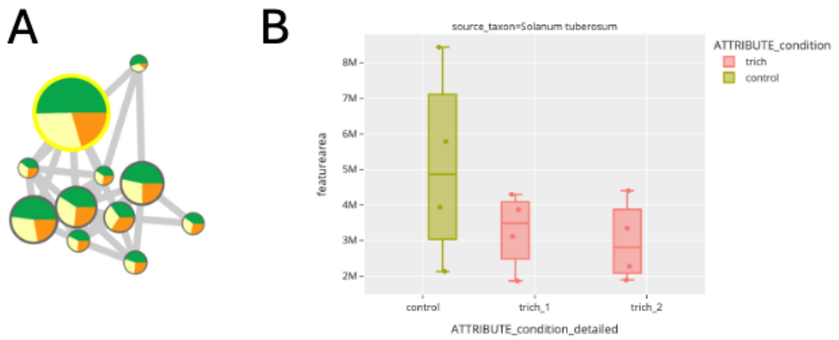
Coumaric acids play a fundamental role in plant defences (Liu, Y. *et al.* 2022) by serving as a central precursor for a wide range of bioactive compounds. These include lignins, which contribute to cell wall strengthening and physical barriers against pathogens (Lee, S. O. *et al.* 2010) as well as flavonoids, which are involved in UV protection, antimicrobial activity, and stress signalling.

In the referenced article (Nawrocka, J. *et al.*, 2018), the authors observed that plants treated with *Trichoderma atroviride* TRS25, an endophytic fungus naturally associated with the plant microbiome, showed a significant increase in the accumulation of phenolic compounds. This metabolic response was linked to improved tolerance against *Rhizoctonia solani*, a fungal pathogen, and was interpreted as evidence of broader activation of the plant's chemical defense system (Bouarab, K. *et al.*, 2019). The observed enhancement of phenolic metabolism following *Trichoderma* treatment suggests that these beneficial microbes may stimulate the biosynthesis of aromatic secondary metabolites via shikimate-related pathways, thereby strengthening the plant's natural ability to resist infection.

Interestingly, another study entitled (Purwantisari, S. *et al.* 2018) demonstrated *Trichoderma atroviride*'s (strain SP1) activation of systemic resistance of potato plants against late blight caused by *Phytophthora infestans*. A suspension of this beneficial fungus was applied directly to the soil by watering 14 days prior to planting, and again after planting. The researchers reported increased glucanase activity and higher levels of phenolic compounds in *Trichoderma*-treated plants, which was associated with a significant reduction in disease symptoms. These results are particularly relevant to the present metabolomics analysis, where an increased abundance of phenolic-related traits was also observed in the groups corresponding to *Trichoderma*-treated plants (Fig.

9). This consistency between studies reinforces the interpretation that *Trichoderma* treatment enhances phenolic-based defence responses in plants.

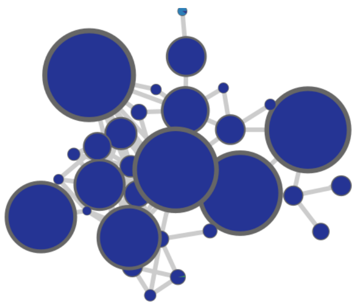
The cluster in Figure 10 supports our observations regarding the increase in phenolic compounds following treatment with *Aspergillus* and *Trichoderma* strains. It consists of precursors of coumaric, as previously described. These phenylpropanoid compounds are more abundant in untreated control plants than in treated ones which supports the idea that, in treated plants, these precursors are actively used to synthesize phenolic compounds, resulting in their lower relative abundance.



C

isdb_structure_taxonomy_npclassifier_02supercla	isdb_structure_taxonomy_npclassifier_	isdb_structure_taxonomy_npclassifier_01pathway_
Phenylpropanoids (C6-C3)	Cinnamic acids and derivatives	Shikimates and Phenylpropanoids
Phenylpropanoids (C6-C3)	Cinnamic acids and derivatives	Shikimates and Phenylpropanoids
Phenylpropanoids (C6-C3)	Cinnamic acids and derivatives	Shikimates and Phenylpropanoids
Phenylpropanoids (C6-C3)	Cinnamic acids and derivatives	Shikimates and Phenylpropanoids
Phenylpropanoids (C6-C3)	Cinnamic acids and derivatives	Shikimates and Phenylpropanoids
Phenylpropanoids (C6-C3)	Cinnamic acids and derivatives	Shikimates and Phenylpropanoids
Phenylpropanoids (C6-C3)	Cinnamic acids and derivatives	Shikimates and Phenylpropanoids
Phenylpropanoids (C6-C3)	Cinnamic acids and derivatives	Shikimates and Phenylpropanoids

Figure 10: **Coumaric acid precursor are less abundant in treated leaflets**. (A) Compounds annotated as part of the superclass of phenylpropanoids, precursors of phenolic compounds such as coumaric acid and its derivatives. (B) The boxplot shows a reduced abundance of on these precursors in treated plants compared to control plants, suggesting their possible conversion into downstream defence-related phenols. (C) Structural annotation of compounds within the cluster are shown.



shared name	ure_exact_mass	isdb_structure_nameTraditional	isdb_IK2D	isdb_npc_pathway	isdb_npc_superclass	isdb_npc_class
1066	284.15247788	Penipaline A	VQSWVFALFFYCMJ	Alkaloids	Tryptophan alkaloids	Carboline alkaloids
1775	258.173213324	2-methyl-4-[3-[2-(methylamino)ethyl]-...	QCWMNSFZHYEJJO	Alkaloids	Tryptophan alkaloids	Simple indole alkaloids
715	254.163042564	1-Hydroxy-3-(1-methoxy-2-methylpr...	SLGUHHKEDMJMIH	Alkaloids	Tetramate alkaloids	Pyrazine and Piperazin...
855	254.163042564	1-Hydroxy-3-(1-methoxy-2-methylpr...	SLGUHHKEDMJMIH	Alkaloids	Tetramate alkaloids	Pyrazine and Piperazin...
477	254.163042564	1-Hydroxy-3-(1-methoxy-2-methylpr...	SLGUHHKEDMJMIH	Alkaloids	Tetramate alkaloids	Pyrazine and Piperazin...
769	238.168127944	Ochramide B	XQDFUPVITSTOSR	Alkaloids	Tetramate alkaloids	Pyrazine and Piperazin...
804	238.168127944	Ochramide B	XQDFUPVITSTOSR	Alkaloids	Tetramate alkaloids	Pyrazine and Piperazin...
653	238.168127944	Ochramide B	XQDFUPVITSTOSR	Alkaloids	Tetramate alkaloids	Pyrazine and Piperazin...
1157	238.168127944	Ochramide B	XQDFUPVITSTOSR	Alkaloids	Tetramate alkaloids	Pyrazine and Piperazin...
709	238.168127944	Ochramide B	XQDFUPVITSTOSR	Alkaloids	Tetramate alkaloids	Pyrazine and Piperazin...
868	238.168127944	Ochramide B	XQDFUPVITSTOSR	Alkaloids	Tetramate alkaloids	Pyrazine and Piperazin...
573	224.15247788	Neoaspergillic acid	VIXDKWMATBSRFJ	Alkaloids	Tetramate alkaloids	Pyrazine and Piperazin...
1247	222.173213324	Nigragillin	DVCNHRTYSUTLOS	Alkaloids	Lysine alkaloids	Piperidine alkaloids
1130	222.173213324	Nigragillin	DVCNHRTYSUTLOS	Alkaloids	Lysine alkaloids	Piperidine alkaloids
409	210.136827816	(3S,8aS)-3-[(2R)-butan-2-yl]-2,3,6,7,8,...	ZDACRNZBFJOLTC	Amino acids and Peptides	Small peptides	Dipeptides
230	210.136827816	(3S,8aS)-3-Isobutylhexahydropyrrolo[1...	SZJNCZMRZAUNQT	Amino acids and Peptides	Small peptides	Dipeptides
1250	208.15756326	Flavacol	UYYRKEKHAYEACU	Alkaloids	Tetramate alkaloids	Pyrazine and Piperazin...
729	208.15756326	Flavacol	UYYRKEKHAYEACU	Alkaloids	Tetramate alkaloids	Pyrazine and Piperazin...
1763	199.099714036	1-(4-benzyl-1H-pyrrol-3-yl)ethanone	VOZPCPOOSANQSB	Alkaloids	Nicotinic acid alkaloids	Pyridine alkaloids
1077	160.100048384	Tryptamine	APJYDQYYACXCRM	Alkaloids	Tryptophan alkaloids	Simple indole alkaloids
2121	146.021523292	Hydron;2-oxopentanedioate	KPGXRSRHYNQIFN	Fatty acids	Fatty Acids and Conjugates	Dicarboxylic acids

Figure 11: **Cluster of fungal metabolites found in the *Aspergillus tubingensis* sample**

A closer look at the fungal metabolites

This cluster (Fig. 11) corresponds to features found almost exclusively in the *Aspergillus* sample. Which is further supported by the presence of annotated fungal compounds such as Neoaspergillic acid, Ochramide B and Nigragillin. The 2 latter molecules belong to various classes of bioactive alkaloids, some of which are known for their antimicrobial or cytotoxic activities (*Zhou et al., 2021*), (Wang et al., 2024)

Ochramide B is a fungal alkaloid from the tetramic acid family, produced notably by *Aspergillus ochraceus*. As a pyrazine-derived compound, it is part of a broader class of secondary metabolites derived from leucine precursors and often associated with antimicrobial or cytotoxic activities (Peng et al., 2018)

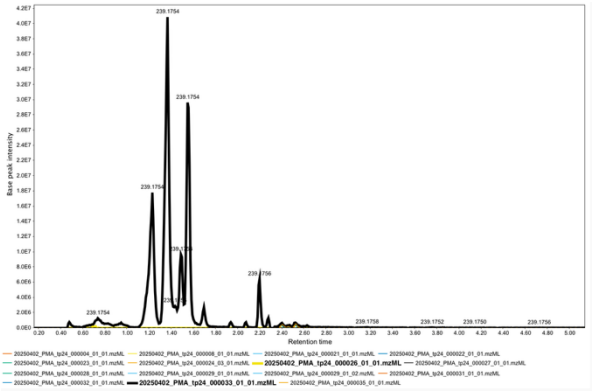


Figure 14 : **Extracted ion chromatogram of Ochramide B.** A compound annotated as Ochramide B, known to be produced by *Aspergillus spp.* (COCONUT), was found in high intensity in the *Aspergillus* sample, confirming the presence of the genus here.

In addition, a compound specific to *Aspergillus tubingensis*, annotated as Tubingensin B, could be observed on the chromatogram of the corresponding sample, confirming the identification of this fungus which was subject to controversy (TePaske et al., 2001).

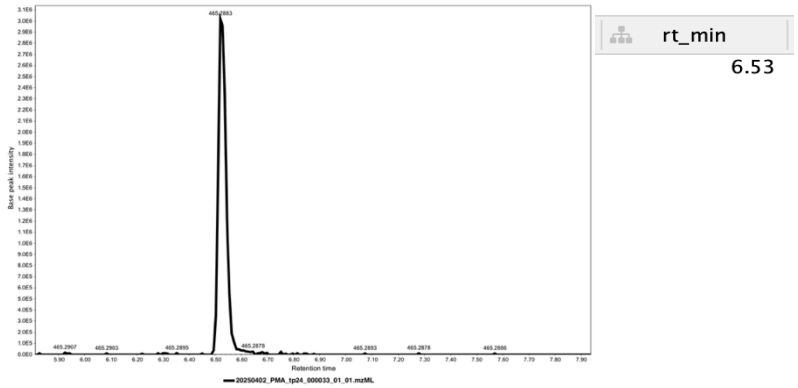


Figure 15: **Extracted ion chromatogram of compound annotated as Tubingensin B.**

Terreusterpene A is a natural meroterpene compound derived partly from the terpene pathway and partly from other biosynthetic pathways (often polyketidic) (Amr, K. *et al.* 2023). It was first isolated from *Aspergillus terreus*, a species known for its rich diversity of bioactive secondary metabolites (*Qi et al., 2023*). The detection of Terreusterpene A in almost all samples (plants and fungi confounded) may reflect structural similarities with

related metabolites, or a partial spectral match in MS/MS annotation. However, its much higher abundance in *Aspergillus*-treated plants suggests that it is produced or induced by this strain. Although no antimicrobial activity has been reported so far, Terreusterpene A has shown inhibitory effects on BACE1 and acetylcholinesterase, enzymes involved in the progression of Alzheimer's disease (Qi et al., 2023).

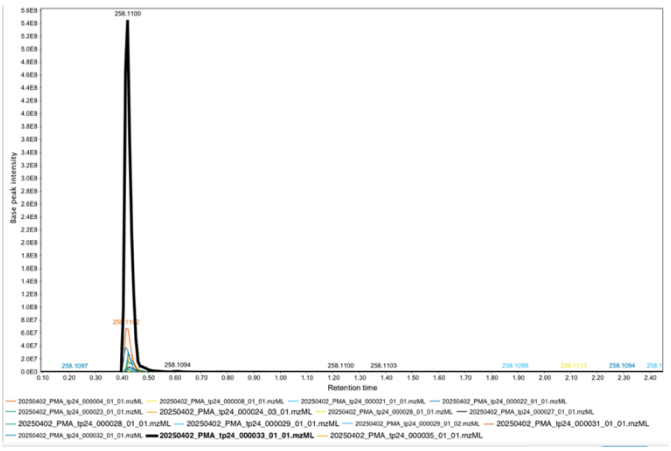


Figure 16 **: Extracted ion chromatogram of Terreusterpene A from *Aspergillus tubingensis* sample**

A compound found abundantly and uniquely in the *Trichoderma* sample, was the main subject of the article entitled "Formation of 6-n-pentyl-2H-pyran-2-one (6-PAP) and other volatiles by different *Trichoderma* species". This study evaluated the potential production of 6-PAP and other volatile compounds by different *Trichoderma* species in relation to their efficacy as biocontrol agents. Antifungal tests showed that 6-PAP strongly inhibits the growth of several *Fusarium* species, including *F. graminearum* and *F. culmorum*. *T. atroviride* was identified as the best 6-PAP producer and a good candidate for biocontrol of phytopathogenic fungi (Jeleń et al., 2014).

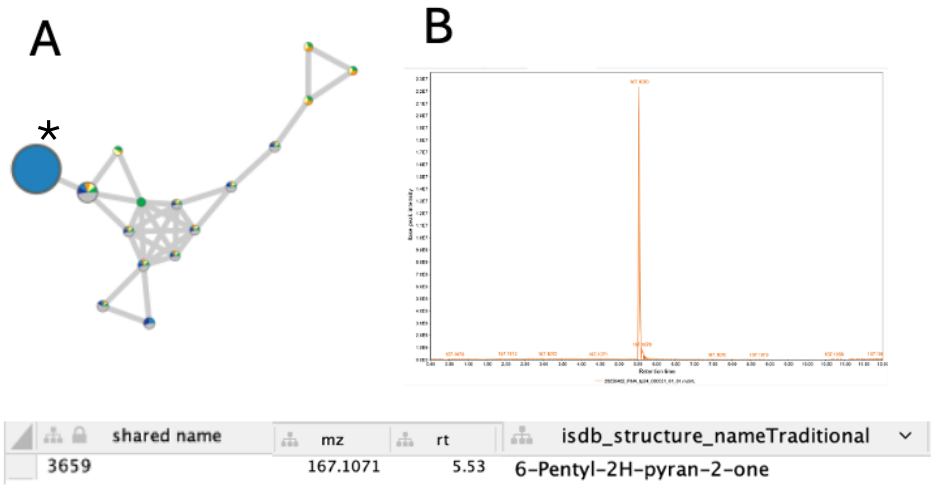


Figure 17: ***Trichoderma atroviride* produces 6-PAP.** (A) Cluster containing a compound (*) annotated as 6-pentyl-2H-pyran-one, specific to *Trichoderma atroviride*. (B) Extracted ion chromatogram of the feature, highlighting its strong signal intensity

Conclusion

Our initial hypothesis, that fungal metabolites would be detectable in treated leaves as evidence of colonization, could not be supported by our findings. Despite thorough comparative analysis, we found no putative metabolite clusters uniquely shared between the fungal samples and the corresponding treated plant samples. This indicates that, under the conditions of our experiment, neither the fungi nor their metabolites established a detectable presence within the leaf tissue.

Our second hypothesis appears to be supported by the data: soil drenching with fungal spore suspensions appears to have triggered a systemic immune response in the plants. This is evidenced by the increased abundance of phenolic compounds and saponins in treated leaflets, both of which are well-established contributors to natural plant defense mechanisms. These metabolomic changes suggest that the fungal treatments may have primed the host plants for defence, potentially enhancing their capacity to respond to subsequent pathogenic challenges.

Importantly, our analyses detected specific metabolites such as monolinolein and phloretin, both of which have previously been documented for their antifungal and antioomycete properties, including specific inhibitory activity against *Phytophthora infestans*. The presence of these defense-related molecules supports the notion that fungal treatments may have triggered a primed or activated state in the plants, potentially enhancing their capacity to resist future pathogen attacks.

Although our main objectives centered on plant metabolic responses and potential fungal colonization, we also independently analyzed the metabolic profiles of *Trichoderma atroviride* and *Aspergillus tubingensis*. This complementary approach provided valuable insight into their unique chemical signatures. Importantly, since we were not completely certain about the identity of our strains prior to this analysis, the metabolomic profiles served as an additional validation: the results aligned well with our previous sequencing data, reinforcing our confidence that the strains used in this study were correctly identified as *T. atroviride* and *A. tubingensis*.

References

- Johnson, D.A., Johnson, S.B., McGrath, M.T., Myers, K.L., Ristaino, J.B., Roberts, P.D., Secor, G., Smart, C.D. (2015). Five Reasons to Consider Phytophthora infestans a Reemerging Pathogen. *Phytopathology*. **105(7):** 966–981. doi:10.1094/PHYTO-01-15-0005-FI
- Coomber, A., Saville, A. & Ristaino, J.B. (2024). Evolution of Phytophthora infestans on its potato host since the Irish potato famine. *Nature Communications*. **15:** 6488. doi:10.1038/s41467-024-50542-5
- Sheoran, A.R., Lakra, N., Saharan, B.S. et al. (2025). Enhancing Plant Disease Resistance: Insights from Biocontrol Agent Strategies. *Journal of Plant Growth Regulation*. **44:** 436–459.
- de Vrieze, M., Gloor, R., Codina, J.M., Torriani, S., Gindro, K., L'Haridon, F., Bailly, A. & Weisskopf, L. (2019). Biocontrol activity of three pseudomonas in a newly assembled collection of phytophthora infestans isolates. *Phytopathology*. **109(9):** 1555–1565. doi:10.1094/PHYTO-03-19-0079-R
- Ebrahimi-Zarandi, M., Saberi Riseh, R., Tarkka, M.T. (2022). Actinobacteria as Effective Biocontrol Agents against Plant Pathogens, an Overview on Their Role in Eliciting Plant Defense. *Microorganisms*. **10(9):** 1739. doi:10.3390/microorganisms10091739
- Madlhophe, S., Ogugua, U.V., Makhubu, F.N. et al. (2025). Use of biological control agents for managing fungal pathogens in Solanaceae crops: progress and future perspectives—a review. *Discover Applied Sciences*. **7:** 83. doi:10.1007/s42452-024-05889-x
- Abada, K.A., El-Nady, A.M.A., El-Nahas, S.E.M., & Zayton, M.A. (2025). Trichoderma as a biocontrol agent for damping-off and its impact on cucumber biochemical alterations. *Italian Journal of Mycology*. **54(1):** 77–97.
- Wang, S., Wang, Y., Shi, X., Herrera-Balandrano, D.D., Chen, X., Liu, F., Laborda, P. (2024). Application and antagonistic mechanisms of atoxigenic Aspergillus strains for the management of fungal plant diseases. *Applied and Environmental Microbiology*. **90:** e01085-24. doi:10.1128/aem.01085-24
- Bao, L., Cai, W., Cao, J., Zhang, X., Liu, J., Chen, H., Wei, Y., Zhuang, X., Zhuang, G., & Bai, Z. (2020). Microbial community overlap between the phyllosphere and rhizosphere of three plants from Yongxing Island, South China Sea. *MicrobiologyOpen*. **9(7):** e1048. doi:10.1002/mbo3.1048
- Agbessenou, K., Biondi, A., Koffi, M.C., Tounou, A.K., Tamo, M., & Tamò, M. (2022). The endophyte Trichoderma asperellum M2RT4 induces the systemic release of methyl salicylate and (Z)-jasmone in tomato plant affecting host location and herbivory of Tuta absoluta. *Frontiers in Plant Science*. **13:** 860309. doi:10.3389/fpls.2022.860309

- Loomans, A.J.M. (2021). Every generalist biological control agent requires a special risk assessment. *BioControl*. **66:** 23–35. doi:10.1007/s10526-020-10058-6
- Hunziker, L., Bönisch, D., Groenhagen, U., Bailly, A., Schulz, S., & Weisskopf, L. (2015). Pseudomonas strains naturally associated with potato plants produce volatiles with high potential for inhibition of Phytophthora infestans. *Applied and Environmental Microbiology*. **81(3):** 821–830. doi:10.1128/AEM.02999-14
- de Vrieze, M., Germanier, F., Vuille, N., & Weisskopf, L. (2018). Combining Different Potato-Associated Pseudomonas Strains for Improved Biocontrol of Phytophthora infestans. *Frontiers in Microbiology*. **9:** 2573. doi:10.3389/fmicb.2018.02573
- Yao, Y., Li, Y., Chen, Z. et al. (2016). Biological Control of Potato Late Blight Using Isolates of Trichoderma. *American Journal of Potato Research*. **93:** 33–42. doi:10.1007/s12230-015-9475-3
- Richard, T., Abdelli-Belhad, A., Vitrac, X., Waffo-Téguo, P., & Mérillon, J.-M. (2016). Vitis vinifera canes, a source of stilbenoids against downy mildew. *OENO One*. **50(3):** (no page numbers available).
- Jin, N., Liu, S.M., Peng, H. et al. (2019). Isolation and characterization of Aspergillus niger NBC001 underlying suppression against Heterodera glycines. *Scientific Reports*. **9:** 591. doi:10.1038/s41598-018-37039-4
- Berendsen, R.L., Vismans, G., Yu, K., Song, Y., De Jonge, R., Burgman, W.P., Burmølle, M., Herschend, J., Bakker, P.A.H.M., Pieterse, C.M.J. (2018). Disease-induced assemblage of a plant-beneficial bacterial consortium. *ISME Journal*. **12:** 1496–1507. doi:10.1038/s41396-018-0092-1
- Trdá, L., Janda, M., Macková, D., Pospíchalová, R., Dobrev, P.I., Burketová, L., & Matušinsky, P. (2019). Dual mode of the saponin aescin in plant protection: Antifungal agent and plant defense elicitor. *Frontiers in Plant Science*. **10:** 1448. doi:10.3389/fpls.2019.01448
- Liu, Y., Liu, X., Li, Y., Pei, Y., Abdul Jaleel, Ren, M. (2024). Potato steroidal glycoalkaloids: properties, biosynthesis, regulation and genetic manipulation. *Molecular Horticulture*. **4:** 43. doi:10.1186/s43897-024-00118-y
- Li, Y., Yang, H., Li, Z., Li, S., Li, J. (2023). Advances in the Biosynthesis and Molecular Evolution of Steroidal Saponins in Plants. *International Journal of Molecular Sciences*. **24(3):** 2620. doi:10.3390/ijms24032620
- Duan, L., Yu, Y., Li, L., & Li, Y. (2023). Steroidal saponins: A review of their biological activities, synthetic pathways and structure–activity relationships. *Frontiers in Pharmacology*. **13:** 9917158. doi:10.3389/fphar.2023.9917158
- Xu, Y., Liu, Y., Hou, Y., et al. (2020). Chemical synthesis of saponins. *Chemical Reviews*. **120(21):** 11271–11341. doi:10.1021/acs.chemrev.0c00248
- Wang, Y., Feng, Y., Yang, X., & Zhang, Y. (2023). Steroidal saponins: From natural resources to synthetic biology. *Frontiers in Plant Science*. **14:** 10343844. doi:10.3389/fpls.2023.1172604

- Milner, S.E., Brunton, N.P., Jones, P.W., O'Brien, N.M., Collins, S.G., & Maguire, A.R. (2011). Bioactivities of glycoalkaloids and their aglycones from Solanum species. *Food Chemistry*. **135(3):** 1456–1471. doi:10.1016/j.foodchem.2012.05.049
- Glotter, E. (1991). Withanolides and related ergostane-type steroids. *Natural Product Reports*. **8(4):** 415–440. doi:10.1039/np9910800415
- Chacón, M.G., Matos, C.S., & Rodríguez, D. (2023). Secondary metabolites, other prospective substances, and alternative approaches that could promote resistance against Phytophthora infestans. *Agronomy*. **13(7):** 1822. doi:10.3390/agronomy13071822
- Moses, T., Papadopoulou, K.K., & Osbourn, A. (2021). Metabolic and functional diversity of saponins, biosynthetic intermediates and semi-synthetic derivatives. *Advances in Botanical Research*. **97:** 1–35. doi:10.1016/bs.abr.2021.01.001
- Shakeel, A., Noor, J.J., Jan, U., Gul, A., Handoo, Z., & Ashraf, N. (2025). Saponins, the unexplored secondary metabolites in plant defense: Opportunities in integrated pest management. *Plants*. **14(6):** 861. doi:10.3390/plants14060861
- Stoessl, A., Fisch, M.H., & Arditti, J. (1980). Monolinolein as a selective fungus inhibitor from Cymbidium, Orchidaceae. *Canadian Journal of Botany*. **53(13):** 2775–2780.
- Larodan. (2019). Monolinolein (Product No. 31-1820; CAS 26545-74-4; Glycerol monolinoleate). Retrieved from https://www.larodan.com/product/monolinolein/
- Zhang, Y., Li, Y., Wang, Y., Wang, X., Wang, W., Wang, J., & Wang, G. (2022). Saponins in the genus Allium: Chemistry, distribution, and biological properties. *Frontiers in Plant Science*. **13:** 1054665. doi:10.3389/fpls.2022.1054665
- Shim, S.-H., Jo, S.-J., Kim, J.-C., & Choi, G.J. (2010). Control Efficacy of Phloretin Isolated from Apple Fruits Against Several Plant Diseases. *The Plant Pathology Journal*. **26(3):** 280–285. doi:10.5423/PPJ.2010.26.3.280
- Purwantisari, S., Priyatmojo, A., Sancayaningsih, R.P., Kasiamdari, R.S., & Budihardjo, K. (2018). Systemic inducing resistance against late blight by applying antagonist Trichoderma Viride. *Journal of Physics: Conference Series*. **1025:** 012053.
- Amr, K., Ibrahim, N., Elissawy, A.M., & Singab, A.N.B. (2023). Unearthing the fungal endophyte Aspergillus terreus for chemodiversity and medicinal prospects: a comprehensive review. *Fungal Biology and Biotechnology*. **10(1):** 6. doi:10.1186/s40694-023-00153-2
- Qi, C., Qiao, Y., Gao, W., Liu, M., Zhou, Q., Chen, C., Lai, Y., Xue, Y., Zhang, J., Li, D., Wang, J., Zhu, H., Hu, Z., Zhou, Y., & Zhang, Y. (2023). New 3,5-dimethylorsellinic acid-based meroterpenoids with BACE1 and AchE inhibitory activities from Aspergillus terreus. *Bioorganic & Medicinal Chemistry Letters*. **88:** 129337. doi:10.1016/j.bmcl.2022.129337

- Chen, L., Li, E., Wu, W., Wang, G., Zhang, J., Guo, X., Xing, F., et al. (2022). The secondary metabolites and biosynthetic diversity from Aspergillus ochraceus. *Frontiers in Chemistry*. **10:** 938626. doi:10.3389/fchem.2022.938626
- Peng, X., Wang, Z., Liu, J., Lin, W., & Liu, H. (2018). New pyrazine derivatives from a marine-derived Aspergillus ochraceus. *Marine Drugs*. **16(9):** 338. doi:10.3390/md16090338
- TePaske, M.R., Gloer, J.B., Wicklow, D.T., & Dowd, P.F. (2001). The structure of tubingensin B: A cytotoxic carbazole alkaloid from the sclerotia of Aspergillus tubingensis. *Tetrahedron Letters*. **42(50):** 8735–8738. doi:10.1016/S0040-4039(01)93829-8
- Jeleń, H.H., Boczek, T., Kowalski, R., & Wąsowicz, E. (2014). Formation of 6-n-pentyl-2H-pyran-2-one (6-PAP) and other volatiles by different Trichoderma species. *Mycological Progress*. **13:** 589–600. doi:10.1007/s11557-013-0942-2